

【CLAIMS】**【Claim 1】**

<281> A fluorescent probe for real-time detection of amplification of nucleic acid, wherein a fluorescent dye of which intensity of fluorescence is varied when the dye is intercalated into a double-stranded nucleic acid, is connected with an oligonucleotide of which base sequence is complementary with at least a part of said nucleic acid.

【Claim 2】

<282> The fluorescent probe according to Claim 1, wherein said fluorescent dye is connected with at least one selected from the 5' end region, the 3' end region, and the middle region of oligonucleotide.

【Claim 3】

<283> The fluorescent probe according to Claim 1, wherein the 3' end of said fluorescent probe is blocked so that replication cannot begin from said 3' end.

【Claim 4】

<284> The fluorescent probe according to Claim 1, wherein said fluorescent dye is selected from the group of Acridine homodimer and derivatives thereof, Acridine Orange and derivatives thereof, 7-aminoactinomycin D and derivatives thereof, Actinomycin D and derivatives thereof, 9-amino-6-chloro-2-methoxyacridine (ACMA) and derivatives thereof, DAPI and derivatives thereof, Dihydroethidium and derivatives thereof, Ethidium bromide and derivatives thereof, EthD-1 and derivatives thereof, EthD-2 and derivatives thereof, Ethidium monoazide and derivatives thereof, Hexidium iodide and derivatives thereof, bisbenzimidazole(Hoechst 33258) and derivatives thereof, Hoechst 33342 and derivatives thereof, Hoechst 34580 and derivatives thereof, hydroxystilbamidine and derivatives thereof, LDS 751 and derivatives thereof, Propidium Iodide(PI) and derivatives thereof and Cy-dyes derivatives.

【Claim 5】

<285> The fluorescent probe according to Claim 1, wherein the fluorescent dye is labeled at said oligonucleotide by being bonded with or being replaced with a

base of said oligonucleotide.

【Claim 6】

- <286> The fluorescent probe according to Claim 1, wherein said fluorescent probe is hybridized with at least a portion of region which is inside the range of one (1) to fifteen (15) bases from the base on which 3' end of said primer is combined.

【Claim 7】

- <287> The fluorescent probe according to Claim 1, wherein the oligonucleotide is composed of ten (10) to forty (40) bases.

【Claim 8】

- <288> The fluorescent probe according to Claim 1, wherein said oligonucleotide comprises the base sequence selected from the group which consists of the Seq. ID Nos. 1 through 22.

【Claim 9】

A real-time detection method of nucleic acid amplification, comprising the steps of:

- <291> i) producing an aqueous buffer which contains a nucleic acid, a pair of primers for amplification of said nucleic acid, a fluorescent probe wherein a fluorescent dye of which intensity of fluorescence is varied when the dye is intercalated into a double-stranded nucleic acid, is connected with an oligonucleotide of which base sequence is complementary with at least a part of said nucleic acid, four(4) kinds of nucleotides and DNA polymerase;
- <292> ii) denaturing said double-stranded nucleic acid into single strands by heating the aqueous buffer prepared in step i) up to 93°C to 96°C;
- <293> iii) annealing said pair of primers with said single strand by cooling the solution obtained in step ii) up to 50°C to 57°C;
- <294> iv) replicating said single-stranded nucleic acid by heating the solution obtained from step iii) up to 70°C to 74°C;
- <295> v) denaturing said replicated nucleic acid into single strands by heating the solution obtained in step iv) up to 93°C to 96°C;
- <296> vi) annealing said fluorescent probe with said single-stranded nucleic acid

by cooling the solution obtained in step v) up to 50°C to 57°C;

<297> vii) measuring an intensity of the fluorescence emitted from the solution obtained in step vi); and

<298> viii) repeating more than one steps iv) through vii).

<299> **【Claim 10】**

<300> The real-time detection methods according to Claim 9, wherein said step vii) is preformed concurrently with the step vi).

【Claim 11】

<301> A detection method of initial amount of a nucleic acid in a sample, comprising the steps of:

<302> i) producing an aqueous buffer which contains a nucleic acid, a pair of primers for amplification of said nucleic acid, a fluorescent probe wherein a fluorescent dye of which intensity of fluorescence is varied when the dye is intercalated into a double-stranded nucleic acid, is connected with an oligonucleotide of which base sequence is complementary with at least a part of said nucleic acid, four(4) kinds of nucleotides and DNA polymerase;

<303> ii) denaturing said double-stranded nucleic acid into single strands by heating the aqueous buffer prepared in step i) up to 93°C to 96°C;

<304> iii) annealing said pair of primers with said single strand by cooling the solution obtained in step ii) up to 50°C to 57°C;

<305> iv) replicating said single-stranded nucleic acid by heating the solution obtained from step iii) up to 70°C to 74°C;

<306> v) denaturing said replicated nucleic acid into single strands by heating the solution obtained in step iv) up to 93°C to 96°C;

<307> vi) annealing said fluorescent probe with said single-stranded nucleic acid by cooling the solution obtained in step v) up to 50°C to 57°C;

<308> vii) measuring an intensity of the fluorescence emitted from the solution obtained in step vi);

<309> viii) repeating more than one steps iv) through vii);

<310> ix) establishing a standard calibration curve which indicates the correlation between the log value of an initial amount of the nucleic acid and a

threshold cycle shown by the performance of above steps i) through viii), by using a sample of which an initial amount of the nucleic acid is known; and
<311> x) detecting an initial amount of the nucleic acid based on the log value which corresponds to the threshold cycle obtained from the performance of said steps i) through viii), by referring to the standard calibration curve obtained in step ix).

【Claim 12】

<312> The detection method according to claim 11, wherein said step vii) is preformed concurrently with the step vi).

【Claim 13】

<313> A composition for the amplification of a nucleic acid which comprises i) a pair of primers for amplification of said nucleic acid; ii) a fluorescent probe wherein a fluorescent dye of which intensity of fluorescence is varied when the dye is intercalated into a double-stranded nucleic acid, is connected with an oligonucleotide of which base sequence is complementary with at least a part of said nucleic acid; iii) DNA polymerase; and iv) four(4) kinds of nucleotides.

【Claim 14】

<315> The composition according to claim 13, wherein said fluorescent dye is connected with at least one selected from the 5' end region, the 3' end region, and the middle region of oligonucleotide.

【Claim 15】

<316> The composition according to claim 13, wherein the 3' end of said fluorescent probe is blocked so that replication cannot begin from said 3' end.

【Claim 16】

<317> The composition according to claim 13, wherein said fluorescent dye is selected from the group of Acridine homodimer and derivatives thereof, Acridine Orange and derivatives thereof, 7-aminoactinomycin D and derivatives thereof, Actinomycin D and derivatives thereof, 9-amino-6-chloro-2-methoxyacridine (ACMA) and derivatives thereof, DAPI and derivatives thereof, Dihydroethidium and derivatives thereof, Ethidium bromide and derivatives

thereof, EthD-1 and derivatives thereof, EthD-2 and derivatives thereof, Ethidium monoazide and derivatives thereof, Hexidium iodide and derivatives thereof, bisbenzimidazole(Hoechst 33258) and derivatives thereof, Hoechst 33342 and derivatives thereof, Hoechst 34580 and derivatives thereof, hydroxystilbamidine and derivatives thereof, LDS 751 and derivatives thereof, Propidium Iodide(PI) and derivatives thereof and Cy-dyes derivatives.

【Claim 17】

<318> The composition according to claim 13, wherein the fluorescent dye is labeled at said oligonucleotide by being bonded with or being replaced with a base of said oligonucleotide.

【Claim 18】

<319> The composition according to claim 13, wherein said fluorescent probe is hybridized with at least a portion of region which is inside the range of one (1) to fifteen (15) bases from the base on which 3' end of said primer is combined.

【Claim 19】

<320> The composition according to claim 13, wherein the oligonucleotide is composed of ten (10) to forty (40) bases.

【Claim 20】

<321> The composition according to claim 13, wherein said oligonucleotide comprises the base sequence selected from the group which consists of the Seq. ID Nos. 1 through 22.

【Claim 21】

<322> The composition according to claim 13, wherein said composition is dried in vacuum.

【Claim 22】

<323> A real-time detection method of the nucleic acid amplification, comprising the steps of:

<324> i) producing an aqueous buffer which contains a nucleic acid, a pair of primers for amplification of said nucleic acid, a primer for reverse transcription, a fluorescent probe wherein a fluorescent dye of which

intensity of fluorescence is varied when the dye is intercalated into a double-stranded nucleic acid, is connected with an oligonucleotide of which base sequence is complementary with at least a part of said nucleic acid, four(4) kinds of nucleotides, DNA polymerase and reverse transcriptase;

- <325> ii) replicating a single-stranded cDNA by heating the aqueous buffer prepared in step i) up to 42°C to 50°C;
- <326> iii) denaturing a primer for a reverse transcription and a reverse transcriptase from said single-stranded cDNA by heating the solution obtained from said step ii) up to 93°C to 96°C;
- <327> iv) annealing the pair of primers with said single-stranded nucleic acid by cooling the solution obtained from said step iii) up to 50°C to 57°C;
- <328> v) replicating said single-stranded nucleic acid by heating the solution obtained from step iv) up to 70°C to 74°C;
- <329> vi) denaturing said replicated nucleic acid into single strands by heating the solution obtained from step v) up to 93°C to 96°C;
- <330> vii) annealing said fluorescent probe with said single-strand nucleic acid by cooling the solution obtained from step vi) up to 50-57°C;
- <331> viii) measuring an intensity of the fluorescence emitted from the solution obtained in step vii); and
- <332> ix) repeating more than one steps v) through viii).

【Claim 23】

- <333> The detection method according to Claim 22, wherein said step viii) is preformed concurrently with the step vii).

【Claim 24】

- <334> A detection method of the initial amount of a nucleic acid in a sample, comprising the steps of:
- <335> i) producing an aqueous buffer which contains a nucleic acid, a pair of primers for amplification of said nucleic acid, a primer for reverse transcription, a fluorescent probe wherein a fluorescent dye of which intensity of fluorescence is varied when the dye is intercalated into a double-stranded nucleic acid, is connected with an oligonucleotide of which

base sequence is complementary with at least a part of said nucleic acid, four(4) kinds of nucleotides, DNA polymerase and reverse transcriptase;

- <336> ii) replicating a single-stranded cDNA by heating the aqueous buffer prepared in step i) up to 42°C to 50°C;
- <337> iii) denaturing a primer for a reverse transcription and a reverse transcriptase from said single-stranded cDNA by heating the solution obtained from said step ii) up to 93°C to 96°C;
- <338> iv) annealing the pair of primers with said single-stranded nucleic acid by cooling the solution obtained from said step iii) up to 50°C to 57°C;
- <339> v) replicating said single-stranded nucleic acid by heating the solution obtained from step iv) up to 70°C to 74°C;
- <340> vi) denaturing said replicated nucleic acid into single strands by heating the solution obtained from step v) up to 93°C to 96°C;
- <341> vii) annealing said fluorescent probe with said single-strand nucleic acid by cooling the solution obtained from step vi) up to 50-57°C;
- <342> viii) measuring an intensity of the fluorescence emitted from the solution obtained in step vii);
- <343> ix) repeating more than one steps v) through viii);
- <344> x) establishing a standard calibration curve which indicates the correlation between the log value of an initial amount of the nucleic acid and a threshold cycle shown by the performance of above steps i) through ix), by using a sample of which an initial amount of the nucleic acid is known; and
- <345> xi) detecting an initial amount of the nucleic acid based on the log value which corresponds to the threshold cycle obtained from the performance of said steps i) through ix), by referring to the standard calibration curve obtained in step ix).

【Claim 25】

- <346> The detection method according to claim 24, wherein said step viii) is preformed concurrently with the step vii).

【Claim 26】

- <347> A composition for the amplification of a nucleic acid which comprises i) a

pair of primers for amplification of said nucleic acid; ii) a primer for reverse transcription iii) a fluorescent probe wherein a fluorescent dye of which intensity of fluorescence is varied when the dye is intercalated into a double-stranded nucleic acid, is connected with an oligonucleotide of which base sequence is complementary with at least a part of said nucleic acid; iv) DNA polymerase; v) a reverse transcriptase and iv) four(4) kinds of nucleotides.

【Claim 27】

<348> The composition according to claim 26, wherein said fluorescent dye is connected with at least one selected from the 5' end region, the 3' end region, and the middle region of oligonucleotide.

【Claim 28】

<349> The composition according to claim 26, wherein the 3' end of said fluorescent probe is blocked so that replication cannot begin from said 3' end.

【Claim 29】

<350> The composition according to claim 26, wherein said fluorescent dye is selected from the group of Acridine homodimer and derivatives thereof, Acridine Orange and derivatives thereof, 7-aminoactinomycin D and derivatives thereof, Actinomycin D and derivatives thereof, 9-amino-6-chloro-2-methoxyacridine (ACMA) and derivatives thereof, DAPI and derivatives thereof, Dihydroethidium and derivatives thereof, Ethidium bromide and derivatives thereof, EthD-1 and derivatives thereof, EthD-2 and derivatives thereof, Ethidium monoazide and derivatives thereof, Hexidium iodide and derivatives thereof, bisbenzimidazole(Hoechst 33258) and derivatives thereof, Hoechst 33342 and derivatives thereof, Hoechst 34580 and derivatives thereof, hydroxystilbamidine and derivatives thereof, LDS 751 and derivatives thereof, Propidium Iodide(PI) and derivatives thereof and Cy-dyes derivatives.

【Claim 30】

<351> The composition according to claim 26, wherein the fluorescent dye is labeled at said oligonucleotide by being bonded with or being replaced with a base of said oligonucleotide.

【Claim 31】

<352> The composition according to claim 26, wherein said fluorescent probe is hybridized with at least a portion of region which is inside the range of one (1) to fifteen (15) bases from the base on which 3' end 5' end primer is combined.

【Claim 32】

<353> The composition according to claim 26, wherein the oligonucleotide is composed of ten (10) to forty (40) bases.

【Claim 33】

<354> The composition according to claim 26, wherein said oligonucleotide comprises the base sequence selected from the group which consists of the Seq. ID Nos. 1 through 22.

【Claim 34】

<355> The composition according to claim 26, wherein said composition is dried in vacuum.

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